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Neuromuscular junction formation in Extraocular muscles of embryonic mice.

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by

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Abstract

Proper functioning of the visual system depends on correct functioning of the oculomotor system. When the oculomotor system functions correctly, cranial nerves III, IV, and VI migrate to and properly innervate the appropriate extraocular muscles. If proper innervation does not take place, congenital cranial dysinnervation disorders result. One of these disorders is strabismus, a misalignment of the eyes. Despite the importance of the oculomotor system, little is known about the migration and innervation of extraocular muscles embryonically in mice. When a nerve innervates a muscle, a chemical synapse, referred to as a neuromuscular junction (NMJ), is formed. To pinpoint the time of innervation the cranial nerves and NMJs of extraocular muscles were labelled using immunohistochemistry and bungarotoxin between E11.5-E14.5. Extraocular muscles exist as a mass of precursor cells near the eye between E11.5 and E12.5. This precursor mass is innervated by a nerve plexus. By E13.5, the precursor cell mass has segregated and muscles have reached their approximate positions around the eye. By this point each muscle is innervated by its respective cranial nerve. NMJs are visible at E13.5 suggesting they form between E12.5 and 13.5. And at E14 extraocular muscles have reached their final positions around the eye, and NMJs show distinct patterning.

Acknowledgements

I would like to thank Dr. Grant Mastick, for mentoring me during the length of this project and overall during my time in the lab. I would also like to express thanks to the entire Mastick lab including Katie Weller, Tatiana Fontelonga, and Minkyung Kim for their assistance and guidance in the lab. Lastly, the office of undergraduate and interdisciplinary research for awarding me the General Undergraduate Research Grant to help fund this project.

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Introduction

When light passes through the eye and hits the retina, it is transmitted to neural activity. This information is distributed throughout the visual system to create the image of the world we perceive around us. This seamless image is a result of many intricate processes including visual acuity, depth perception, tracking, and a stable image being present on the retina. The oculomotor system is involved in movement of the eyeball and eyelid and thus the field of view. Without the proper functioning of the oculomotor system, the processes so integral to vision would not be able to occur. This eye movement is carried out by six extraocular muscles (EOMs) (figure 1), which are under the control of three cranial nerves: the abducens, trochlear, and oculomotor. The six extraocular muscles work together to control eye movement in three antagonistic pairs, the superior and inferior oblique controlling rotational movement, the lateral and medial rectus guiding horizontal eye movement, and the superior and inferior rectus controlling vertical eye movements (Chilton & Guthrie, 2004). Four of the six extraocular muscles: the superior rectus, inferior rectus, inferior oblique, and the medial rectus, are innervated by the oculomotor nerve, making it the dominant nerve of the oculomotor system. The abducens nerve innervates the lateral rectus, and the trochlear nerve innervates the superior oblique.

Eye movements depend on the proper development of the oculomotor system in the embryo; this involves the correct migration of cranial motor axons and innervation of their corresponding extraocular muscles. Defective innervation can lead to congenital cranial dysinnervation disorders including strabismus, or misalignment of the eye.

Treatment in childhood can correct, or improve eye alignment; however, if treatment is not carried out normal eye functioning, and thus normal vision, are impacted. Depth perception is shown to be impaired even in the case of micro-strabismus (Distler, & Hoffmann, 1991). Vision is further altered due to two conflicting images being sent to the brain from the eyes. When strabismus occurs there is also a greater incidence of nystagmus (excessive slow eye movements), saccadic intrusions (excessive rapid eye movements) and drifting of the eyes (Ciuffreda, 1979). Strabismus can also cause the brain to focus on input from one eye, while signals from the other eye are suppressed causing the eye to become amblyopic, and have a very low visual acuity, 20/200 or less (Attebo et al., 1998) and can even lead to partial blindness.

The oculomotor and trochlear nerves arise from the ventral mesencephalon (midbrain), while the abducens nerve originates from the pons (Laine, 1996). Extraocular muscles that are innervated by the oculomotor nerve (the superior rectus, inferior rectus, lateral rectus, and inferior oblique) are derived from cells in the prechordal mesoderm (Noden & Francis-West, 2006). While the medial rectus and superior oblique originate from paraxial mesoderm. These precursor cells form masses, or primordia, which migrate to the periocular region and around the eye to their final locations by unknown mechanisms (Wahl et al., 1994). The lateral rectus' innervation pattern is unique among the extraocular muscles; the abducens nerve contacts the lateral rectus while it is still a precursor cell mass in the paraxial mesoderm (Wahl et al., 1994) and the two migrate together to the eye.

As the oculomotor nerve migrates out of the midbrain and innervates the extraocular muscles it, like all other migrating axons, is guided by molecular mechanisms, or guidance cues. These guidance cues attract or repel growth cones and therefore guide axons to their correct muscle. Since Slit/Robo signaling is implicated as an important factor in the development of a number of different motor neuron populations (Bravo-Ambrosio et al., 2012) (Murray et al., 2010), these signals are highly probable candidates for the development of the oculomotor system. Slits are a secreted glycoprotein, which typically act as a repellent signal by engaging its Robo receptors (Brose et al., 1999), and are a probable candidate for the predominant guidance cue for oculomotor migration. Although the role of Slit/Robo signaling has been shown to repel some hindbrain motor neurons (Hammond et al., 2005) its role on oculomotor neurons has not been addressed. It has also been hypothesized that the extraocular muscles themselves are providing cues that guide their respective nerves. Chilton and Guthrie observed that the OMN stops migration between Hamburger Hamilton (HH) stage 20 and begins migration again at HH stage 22. They hypothesized that this could be due to the nerve waiting to receive signals from the embryonic muscle mass (Chilton and Guthrie, 2004). The cranial nerves may also provide essential molecular cues to the developing muscle. When an embryo develops in the absence of trochlear and oculomotor motorneurons, extraocular muscle development is significantly altered (Porter & Baker, 1997).

Despite the importance of the oculomotor system, little is known about the timing of migration and innervation of extraocular muscles in mice. Before the role of molecular

cues on the developing oculomotor system of mice can be investigated, the typical migration and innervation pattern in the organism must be mapped. When a nerve contacts and innervates a muscle, a chemical synapse is formed that involves signaling between motor neurons and muscle cells (Dennis, 1981). This chemical synapse is called a neuromuscular junction (NMJ) (figure 2), in vertebrates acetylcholine is released from the motor neuron and binds to nicotinic acetylcholine receptors present in shallow folds on the post synaptic terminal on the muscle cell (Sanes & Lichtman, 2001). NMJ formation occurs in steps, typically after muscle fibers form, acetylcholine receptors (AChRs) cluster in myofibers, called prepatterning. These sites are the location where motor neurons form synaptic connections (Witzemann, 2006). To differentiate between when a muscle is truly innervated instead of simply being contacted by a nerve, it is essential to assess when the neuromuscular junction forms during development. My plan is to label NMJs in the extraocular muscles of mice to differentiate when innervation occurs. Once the time course of typical innervation is completed, it will allow the effect of molecular cues on migration and innervation to be addressed.

Materials and Methods

Mouse Embryos

All mice used were wild type CD 1 mice purchased from Charles River Laboratories. Mice care and experimentation were compliant with National Institutes of Health Guide for Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the University of Nevada, Reno approved all experimental protocols. Embryonic day 11 (E11.0), E11.5, and E12.5 embryos were obtained via uterine dissection of a maternal mouse killed by CO_2 inhalation and decapitation. E13.5, E14.0, and E14.5 embryos underwent ex vivo perfusion after dissection from a maternal mouse heavily anesthetized using isoflourine.

Immunohistochemistry

Embryos were short fixed in 4% PFA. After fixing the embryos were imbedded in 15% sucrose and gelatin. 20µm section were taken using a cryostat and placed on frost slides. Slides were placed in warm (37°C) PO_4 for approximately one minute, or until all of the embedding material was removed. Slides were blocked quickly, then for 45 minutes with a phosphate buffered saline (PBS) solution (1% bovine serum albumin, .1% triton). Primary antibody rabbit ȝ-tubulin (1:1000 dilution, Jackson) was placed on the slides in a humidified chamber to incubate overnight. The primary antibody was removed from the slides, which were washed with PBS solution 3 times quickly than 4 times for 5 minutes each. The secondary antibody Alexa 488 donkey anti rabbit (1:300 dilution, Jackson) was placed on the slides to incubate in a humidified chamber for four hours. Once the

secondary was removed slides were washed in the same manner as with the primary. Fluorsave was applied and slides were covered with a glass coverslip. Labeling of the antibody was visualized using a fluorescent microscope.

Bungarotoxin

Embryos were short fixed in 4% PFA and embedded in a 15% sucrose gelatin solution. They were sectioned using a cryostat to 20µm thick and placed on frost slides. The sucrose gelatin solution was melted off using warm PO_4. Sections were blocked for 45 minutes in a PBS solution (1% BSA, .1% triton) and bungarotoxin (1:1000 dilution, life technologies) was placed on the slides, which were incubated for one hour in a humidified chamber. After removal of bungarotoxin slides were washed 3 times quickly, and 4 time for 5 minutes each. Fluorsave was placed on the slides which were then covered with a glass cover slip. Visualization of bungarotoxin labelling was done using fluorescence microscopy.

Results

Extraocular muscles exist as mass of precursor cells next to the eye from embryonic day 11.0 to 12.5.

Between embryonic days 11.0 and 12.5 the Extraocular muscles are visible under fluorescent microscopy as a precursor cell mass near the eye (figure 3). This mass is expected given extraocular muscles are known to migrate in a mass of precursor cells to their location around the eye. It is consistent with the finding that between E11-13 most head and face muscle progenitors migrate to near their final locations, where the mass becomes segregated and muscles move to their target locations (Noden & Francis-West, 2006).

At embryonic day 11.5 the extraocular precursor cells mass is visible being contacted by a nerve plexus.

Using immunohistochemistry to label the ȝ-tubulin present in microtubules of nerve fibers, a nerve plexus is visible branching into the primordial muscle mass at E11.5 (figure 4). The muscle precursor mass persists to E12.5 and remains contacted by the branching nerve plexus (figure 4). The nerve plexus belongs to the oculomotor nerve. Simply using immunohistochemistry to visualize the nerve plexus and muscles does not tell us if true innervation has occurred, because innervation is defined as to supply with nervous signaling. The close proximity of the nerve and muscle does not indicate if the muscle cells and motor neurons are communicating with one another.

Extraocular muscles that have reach approximate positions around the eye by E13.5.

Labeling reveals that the precursor muscle mass present at E12.5 has segregated and individual muscle precursor cells have migrated around the eye by E13.5 (figures 5). All six extraocular muscles are visible around the eye at this point. Each muscle is contacted by its respective cranial nerve (figure 5 & 6). The oculomotor nerve is visible innervating the medial rectus and sending branches dorsally and ventrally to its other muscle targets (figure 6).

Neuromuscular junctions are visible on the Extraocular muscles by E13.5.

Neuromuscular junctions are visible by E13.5 suggesting they form sometime between E12.5 and E13.5 (figures 7). The bungarotoxin labels nicotinic acetylcholine receptors present in the NMJ, when bungarotoxin binds to these receptors NMJs become labelled. The labelling appears as bright red punctate points on the muscle. It is thus far unclear if each bright red point is one single NMJ or many NMJs clustered together. When a nerve fiber reaches a muscle it branches into many nerve terminals, each of these terminals forms multiple NMJs. It therefore seems that each punctate point is many NMJs, but it is impossible to be sure until further analysis is completed. For this analysis I examined five of the six extraocular muscles. Because of the orientation of the inferior oblique it is not clearly visible when the embryo is sectioned in a coronal manner; therefore, I was unable to observe NMJ formation on this particular muscle. NMJs were visible on all five muscles examined. I speculate NMJs have also formed on the inferior oblique by E13.5 but further analysis will be needed to confirm this.

Neuromuscular junctions patterning changes between E13.5 and E14.

Extraocular muscles have reached their final positions around the eye by E14. These muscles are innervated by the three cranial nerves respectively. NMJ positioning on the muscles changes significantly between E13.5 and E14. Close up photos of the lateral and medial rectus muscles in figure 7 show that NMJs are present all over the muscle, but are not patterned in any specific way. By E14 the NMJs have lined up in a particular way. The superior oblique in figure 8C shows this patterning, with a band on NMJs present in the middle of the muscle. This is consistent with typical NMJ formation, where NMJs are lined up in a central end-plate band in adult muscles (Sanes and Lichtman, 1999). The explanation for NMJ localization throughout the muscle at E13.5 needs to be further investigated, but the overall process of NMJ formation sheds light on potentially why this is occurring. During development acetylcholine receptors (AChRs) form and reach a uniform density on the developing myotubes. The nerve then sends signals which cause AChR clustering, and repress AChR gene expression where a synapse is not present (Sanes and Lichtman, 1999). Since bungarotoxin labels AChRs it is possible we are observing the before and after period of AChR redistribution in the muscle. Further analysis will need to be done to confirm if this is the case. Additionally, in developing muscle some high density clusters of NMJs form spontaneously without nerve muscle contact. This phenomenon could further explain NMJs that are present at sites where a nerve has not contacted the muscle.

Discussion

OMN plexus contact with the primordial muscle cell mass differs from oculomotor nerve innervation pattern in chicks.

OMN contact with the precursor cell mass behind the eye differs vastly from the innervation pattern observed in embryonic chicks (Chilton and Guthrie, 2001) They used immunohistochemistry in chick embryos to visualize OMN migration. Their results indicated that the OMN does not migrate to its Extraocular muscles until they have segregated from the primodial mass and positioned themselves around the eye at HH 25 (E11.75). My results showed that the OMN contacted the muscles before they segregated from the primodial mass and began to move around the eye.

Migration of extraocular muscles around the eye and contact by cranial nerves further diverges from OMN migration in chick embryos.

In chick embryos the OMN branches first to its most distal target, the ventral oblique, by HH stage 27 (E12.25) then projects branches to the remaining three EOMs it innervates. In mice the OMN does not innervate the inferior oblique first then send branches back to the other three muscles it will innervate. The OMN is part of a plexus that innervates muscle precursor cells at E12.5 and by E13.5 it is visible contacting all of its extraocular muscles. This is consistent however with previous research that there is no delay in contact of EOMs by the OMN in mammals (Fritzsch, 1995).

Neuromuscular junction formation between E12.5 and E13.5 contrasts previous research on the formation of NMJs in rats.

In the superior rectus muscle of rats neuromuscular junctions form in two fiber types by the end of the first post natal week (Nag & Cheng, 1982). This suggests NMJs do not differentiate until the post natal period. Our results suggest NMJ formation occurs much earlier in development a little more than half way through embryonic maturation. Another study in extraocular muscles of rats labelled NSE, a protein component in the synaptic plasma membrane and axons, which is also involved when synapses are formed. At E15 (E13 in mice) NSE was observable in the lateral recuts, inferior rectus, superior rectus, and inferior oblique (all muscles innervated by the OMN). NSE was not shown in the superior oblique until E15 and the medial rectus until E16. Nerve fibers containing NSE suggest a synapse is being formed and therefore that synapse formation occur at E15 in four muscle, and E16 and 17 for the remaining muscles in rats, our findings both approve and disagree with these results. We did find that NMJs form between E12.5-13.5 in the extraocular muscles innervated by the OMN, the same period the NSE appears in these muscles in rats since developmentally E15 rats are equivalent to E13 mice. In the superior oblique NSE appears at E16 (equivalent to E14 in mice) and in the medial rectus at E17 (E15 in mice) suggesting that synapses do not form until this point. However NMJs were shown to form between E12.5-13.5 in the other two muscles not innervated by the OMN, this differs from the NSE formation time period observed in rats; therefore synapse formation was shown to occur .5 and 1.5 embryonic days before previously assumed. It is known that minutes after a nerve contacts a muscle neuromuscular transmission occurs but that fully functioning synapses that more time to form (Kidokoro & Yeh 1982, Chow & Poo 1985, Xie & Poo 1986, Evers et al 1989). This could mean that signals are being transmitted before the formation of the neuromuscular junctions

and this is why NMJs do not form right when the nerve contacts the muscle precursor at E11.5-12.5.

Figures

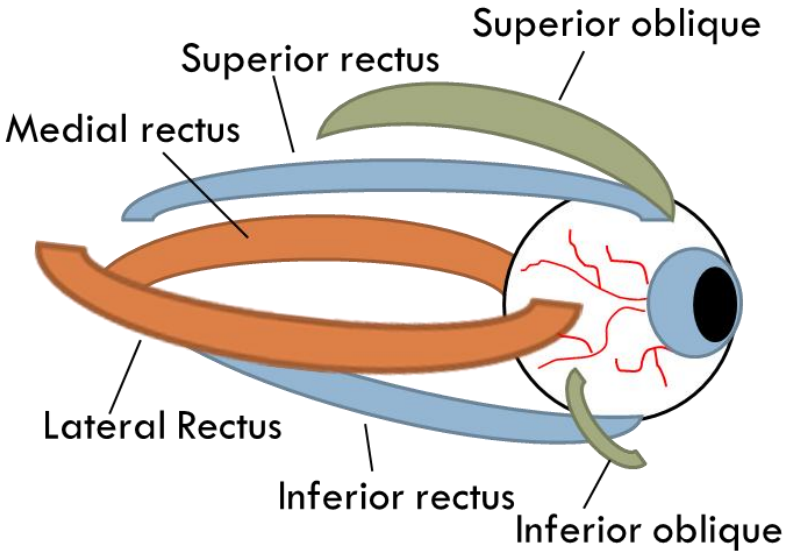

Figure 1: The oculomotor system involves six extraocular muscles. The six Extraocular muscles of the oculomotor system work in three antagonistic pairs. The blue muscles control vertical eve movement, the orange muscles control horizontal movement, and the green muscles control rotational movement.

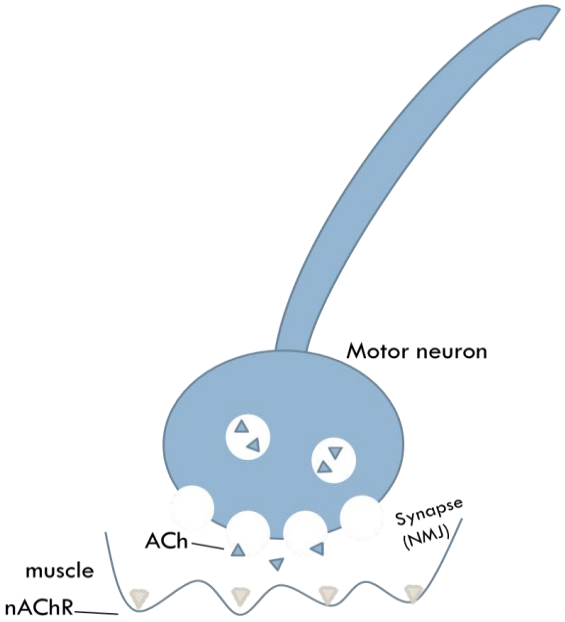

Figure 2: The vertebrate neuromuscular junction. In vertebrates acetylcholine (Ach) is released from the motor neuron and binds to nicotinic acetylcholine receptors present in shallow folds on the postsynaptic side of the NMJ.

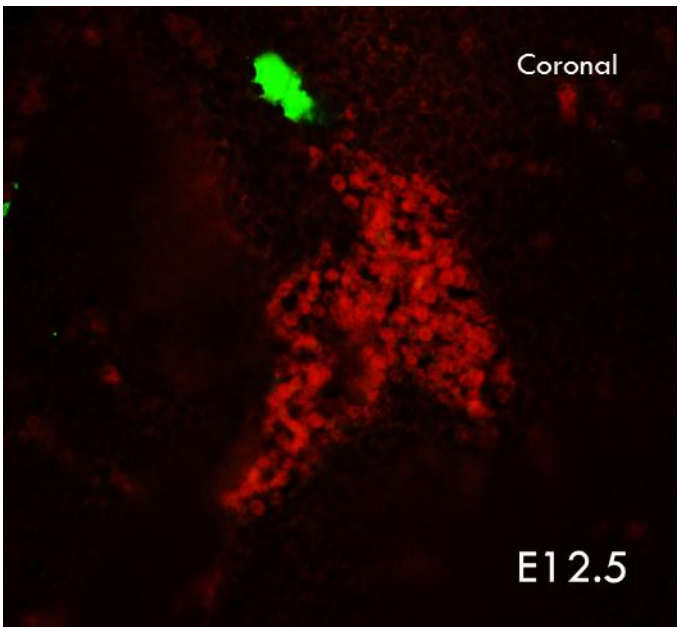

Figure 3: A mass of precursor cells is present behind the eye at E12.5. From E11.5-E12.5 extraocular muscles exist as a mass of precursor cells near the eye.

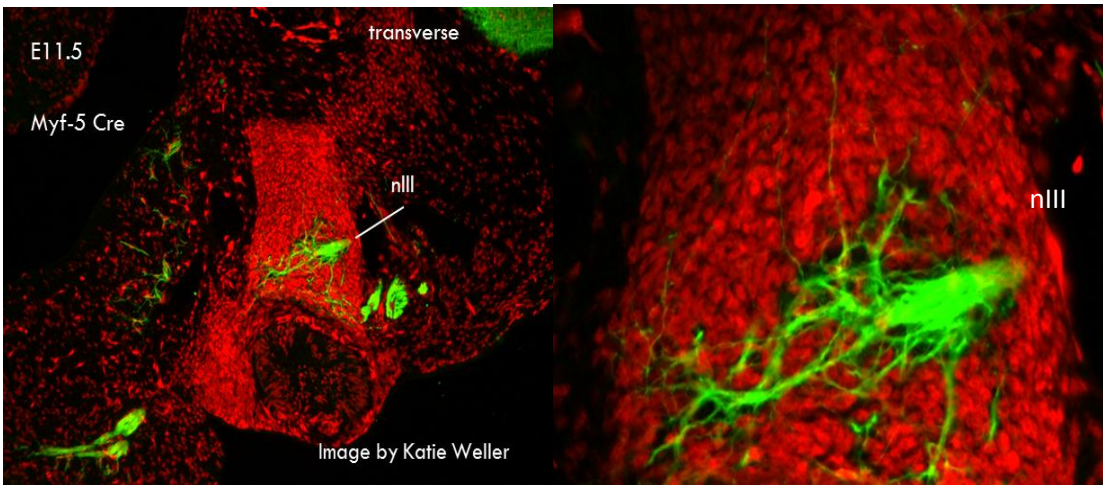

Figure 4**: The precursor cell mass present medial to the eye from E11.5-12.5 is innervated by a nerve plexus.** The plexus innervating the precursor cell mass is the oculomotor nerve. This section was taken from a myf-5 Cre mouse, a mutant mouse engineered to have its myoblast progenitor cells fluoresce. The nerve is visible in green, labelled with ȝ-tubulin.

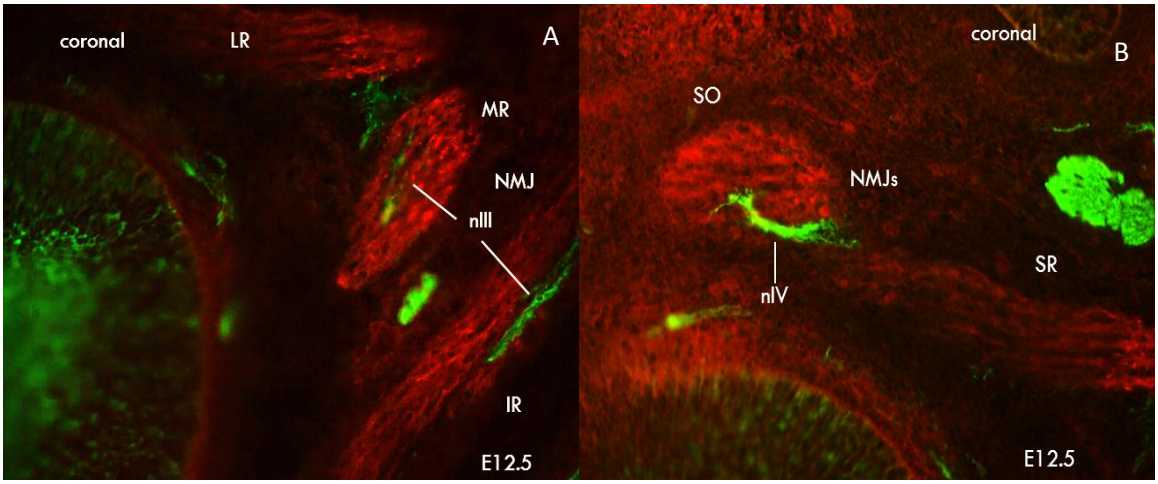

Figure 5: By E13.5 extraocular muscles have reached approximate positions around the eye. Between E12.5-13.5 the mass of precursor cells divides into individual muscles. These individual muscles then move around the eye to their approximate target locations. A shows the medial (MR) and inferior rectus (IR) being innervated by the (nIII). The bottom of the lateral rectus (LR) is also visible. In image B the superior oblique (SO) is visible being innervated by the trochlear nerve (nIV).

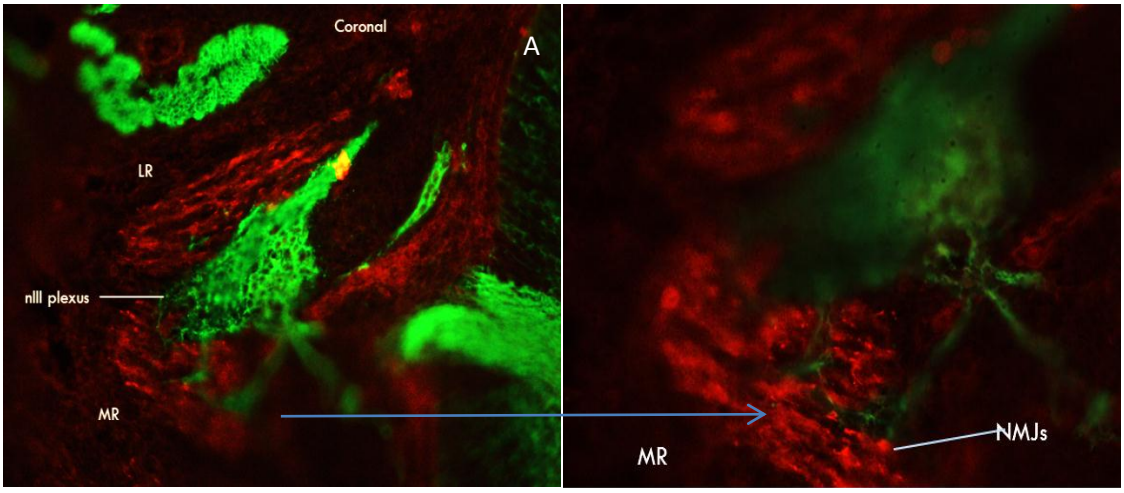

Figure 6: Neuromuscular junctions are visible at E13.5. Nicotinic acetylcholine receptors are labelled at E13.5 suggesting the NMJs form sometime between E12.5 and E13.5. It is unclear when in this time frame the NMJs form, whether it is after segregation from the precursor mass, during movement to their positions, or once they reach their approximate positions. A shows the medial and lateral rectus as well as an oculomotor nerve plexus. B is a magnified version of A showing the medial rectus being contacted by nIII. The bright red points visible throughout the muscle are the neuromuscular junctions.

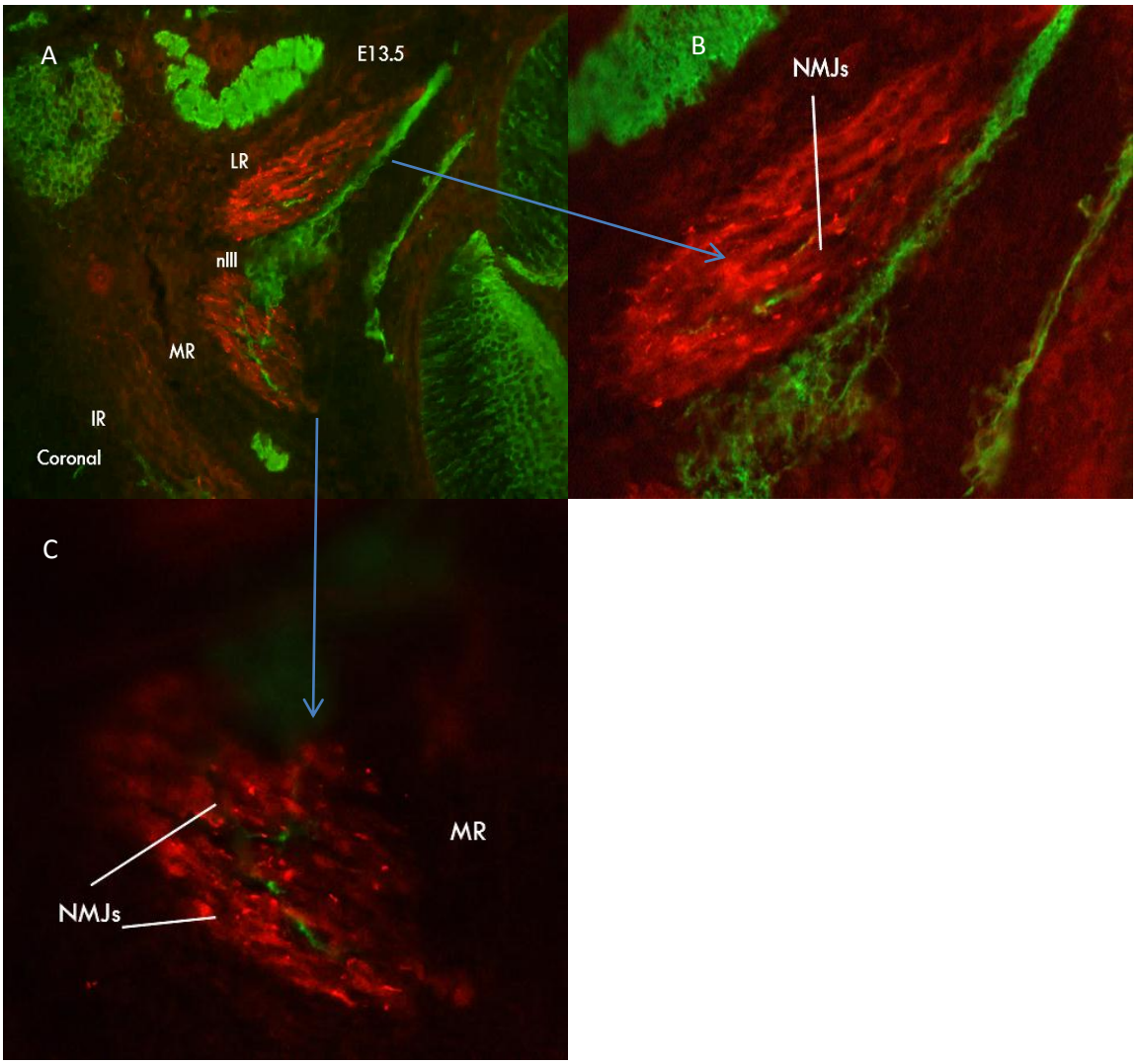

Figure 7: At E13.5 all Extraocular muscles are innervated by their respective cranial nerves. By E13.5 when the muscles have reached their approximate positions around the eye they are visibly innervated by either cranial nerve III, IV, or VI. The OMN is shown sending branches dorsally and ventrally. A shows the medial and lateral rectus as well as the oculomotor nerve. B is a magnified image of the lateral rectus, c is a magnified image of the medal rectus being contacted by the oculomotor nerve.

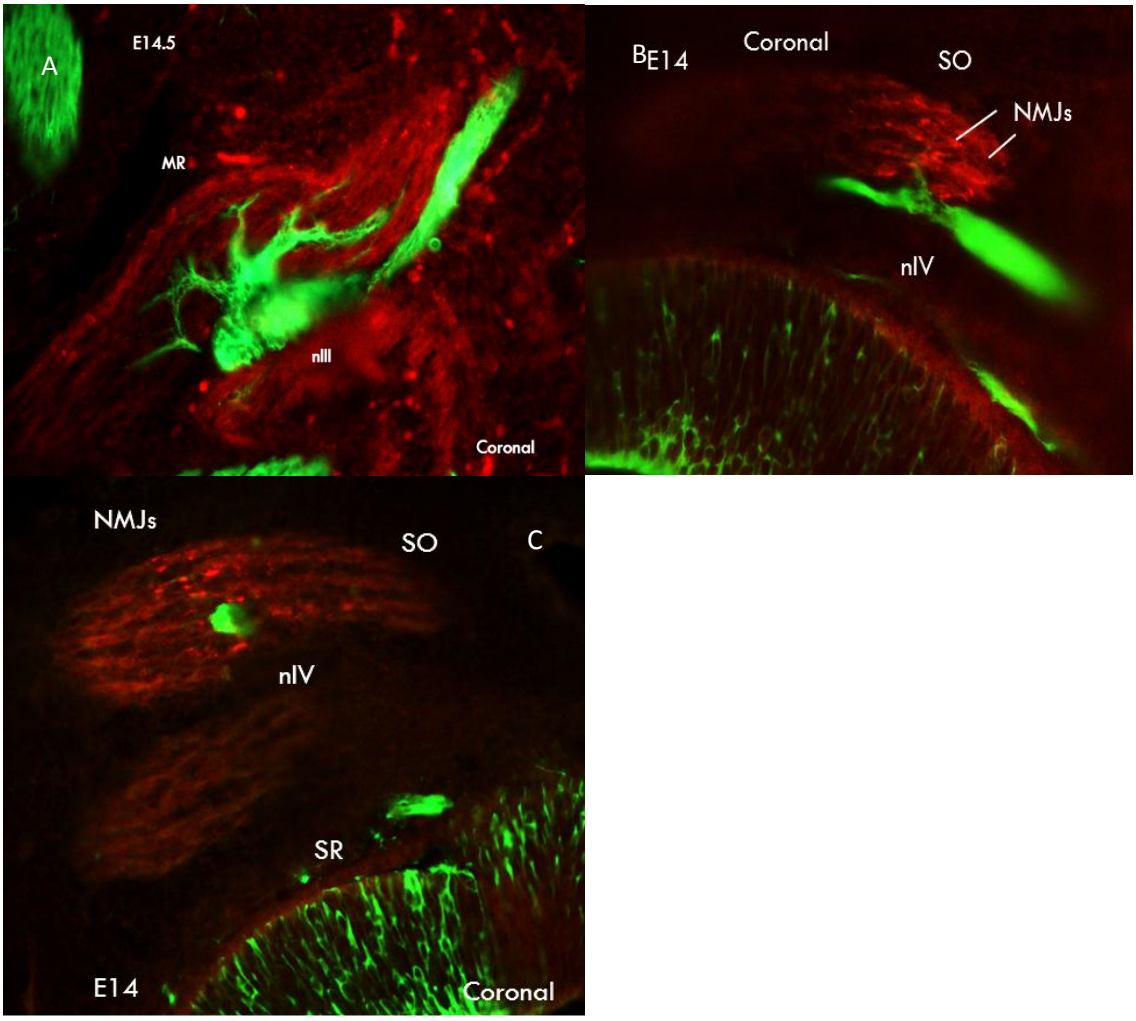

Figure 8: Muscles occupy their final positions by E14. At E14 all muscles occupy their final positions around the eye. These muscles are all innervated by their respective nerves. NMJs are shown lined up in the central end plate region of the superior oblique in C. A shows the medial rectus innervated by the oculomotor nerve. B shows the superior oblique innervated by the trochlear nerve. The superior oblique in image C shows NMJs have lined up in the central region of the muscle.

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